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(54) Title: COMPOSITIONS AND METHODS FOR AMPLIFICATION OF HUMAN STEM CELLS

(57) Abstract: The present invention relates to compositions and methods for amplification of human stem cells. The invention discloses methods using genetically modified donor cells that express a HOXB4 polypeptide and cause enhanced expansion of most primitive stem cells. The invention also relates to compositions comprising such expanded stem cells as well as to their uses, for restoring or stimulating stem cell function or repopulation in a subject. The methods can be used for therapy of various conditions, for tissue regeneration, as well as for research purposes. The invention is particularly suited for expansion of human stem cells, particularly human hematopoietic stem cells.

COMPOSITIONS AND METHODS FOR AMPLIFICATION OF HUMAN STEM CELLS

The present invention relates to compositions and methods for amplification of stem cells. The invention discloses methods using genetically modified donor cells that express a HOXB4 polypeptide and cause enhanced expansion of most primitive stem cells. The invention also relates to compositions comprising such expanded stem cells as well as to their uses, for restoring or stimulating stem cell function or repopulation in a subject. The methods can be used for therapy of various conditions, for tissue regeneration, as well as for research purposes. The invention is suited for expansion of any kind of human stem cells, particularly human hematopoietic stem cells.

Due to their capacity to differentiate into most mature cell types, stem cells offer great therapeutic potential. The ability to produce, expand and maintain such cells ex vivo or in vitro, as well as to control their differentiation, would be of great benefit in the area of tissue repair, cell therapy, protein production, etc. As an example, a major challenge in the field of haematopoietic diseases and cancer is to obtain a sufficient number of purified pluripotent haematopoietic stem cells (HSCs).

Stem cells represent a very rare population able to either long-term self-renew or differentiate according to body needs[1,2]. As a consequence, their use in therapeutic protocols, including cell engineering for transplantation or gene therapy, requires methods allowing their *ex vivo* expansion without loss of their pluripotentiality. Up to the present, *ex vivo* stem cell expansion protocols involve the use of cytokine mixtures whose potency in the maintenance of their biological properties remains controversial since the absence of an irreversible differentiation process cannot be ruled out during culture. An alternative approach consists in using retrovirus-mediated transfer of genes coding for proteins that could lead to their expansion. In this regard, retrovirus-mediated over-expression of proteins in murine bone marrow cells has been reported, leading to an enhanced expansion of hematopoietic stem cells[3-6]. However, even if no leukaemia is detected in mice, such a permanent genetic alteration of the target cells could be harmful in the perspective of a therapeutic use. Consequently, expansion of human stem cells requires alternative approaches.

The present invention now discloses novel approaches to the amplification of stem cells. The proposed methods avoid both genetic alteration of the target cells and the potential drawbacks of cytokine mixtures. The present invention proposes to use genetically modified donor cells to supply a biologically active human HOXB4 polypeptide to the stem cells for expansion. The present invention now shows, for the first time, that the HOXB4 protein can efficiently translocate passively into human stem cells. The present invention also shows, for the first time, that biologically active HOXB4 protein may be produced by donor cells co-cultivated with stem cells, leading to efficient expansion of most primitive cells. The invention further demonstrates, for the first time, that immature human stem cells can be efficiently amplified in the absence of added cytokines using such genetically modified donor cells.

The examples contained in this application show that a functional human HOXB4 protein (hHOXB4) can passively enter human hematopoietic cells. Using *in vitro* long-term culture (LTC) assays and *in vivo* engraftment assays in NOD-SCID immunodeficient mice, we also show that the translocated hHOXB4 protein favours expansion of the most primitive haematopoietic cell populations identifiable in humans: the long-term culture initiating cells (LTC-ICs) and NOD-SCID mouse repopulating cells (SRCs). These results open perspectives for the design of novel and potentially harmless *ex vivo* expansion conditions for human stem cells.

An object of this invention thus resides in a method of amplifying stem cells ex vivo or in vitro, the method comprising co-culturing stem cells with a population of donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide.

An other object of this invention resides in a method of enhancing amplification of stem cells, the method comprising co-culturing, ex vivo or in vitro, stem cells with a population of donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide, said human HOXB4 polypeptide causing enhanced amplification of stem cells.

A further object of this invention resides in a composition of matter comprising stem cells and donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide. The stem cells and donor cells may be present in admixture (i.e., in direct physical contact or interaction), or they may be physically separated in said co-culture, typically by a membrane allowing exchange of soluble factors.

In a specific embodiment, the composition comprises human hematopoietic stem cells and stromal cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide.

A further aspect of this invention is a method of producing a pharmaceutical composition comprising stem cells, the method comprising (i) amplifying stem cells as described above, (ii) isolating amplified stem cells and (iii) conditioning said cells in a pharmaceutically acceptable excipient or carrier.

Still an other object of this invention resides in a method of increasing the stem cell repopulating activity in a human subject, the method comprising (i) collecting a biological sample comprising stem cells from the subject, (ii) co-culturing, ex vivo or in vitro, said stem cells with a population of donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide, under conditions allowing expansion of said stem cells and (iii) administering to the subject said expanded stem cells, said expanded stem cells having increased repopulating activity in said subject.

Other aspects of this invention include :

- a recombinant donor cell, wherein said donor cell is a mammalian adherent cell capable of forming monolayers in culture, and wherein said donor cell comprises a recombinant nucleic acid encoding a human HOXB4 polypeptide, and

- a recombinant lentivirus, wherein said lentivirus encodes a human HOXB4 polypeptide.

Furthermore, because the invention shows, for the first time, that a functional HOXB4 polypeptide can passively translocate into human stem cells and enhance amplification of these cells in culture, the amplification methods according to the present application may also be implemented using an isolated HOXB4 polypeptide instead of donor cells.

The invention can be used for restoring or stimulating stem cell function or repopulation in a subject, in the context of therapy of various conditions, for tissue regeneration, as well as for research purposes.

Within the context of the present invention, the term "stem cell" designates a pluripotent cell capable of self-regeneration and giving rise to progenitor cells that can further differentiate into specific cell lineages. Stem cells may be of various types, including, without limitation, hematopoietic stem cells, mesenchymal stem cells, neuronal stem cells, muscle stem cells (including skeletal muscle, smooth muscle or cardiac muscle stem cells), gut epithelial stem cells, hepatic stem cells, pancreatic stem cells and skin stem cells. Most preferred stem cells are of human origin, and in particular human hematopoietic stem cells. Stem cells may be obtained or isolated from any known human variety of sources, including embryonic or adult tissues. Hematopoietic stem cells may be isolated from umbilical cord blood, bone marrow, peripheral blood, etc. Other sources of stem cells include fetal liver or spleen, embryonic yolk sac, fetal para-aortic region (AGM), somatic tissues such as skin, muscle, etc. Techniques suitable for isolating and identifying human stem cells are well known in the art and include, for instance, tissue collection (e.g. cord blood, cytapheresis, biopsy, etc.), cell culture under appropriate conditions and stem cell separation and characterization, using specific markers. Hematopoietic stem cells are phenotypically heterogenous. Some hematopoietic stem cells express the CD34 antigen and are devoid of CD38 marker antigen and of lineage specific marker. Some immature stem cells are devoid of any specific marker including

the CD34 antigen[42, 43]. One defining property of hematopoietic stem cells (HSC) is low fluorescence after staining with fluorescent dyes such as rhodamine 123[44] and Hoechst 33342 (Hoechst)[45]. A recently developed technique employing Hoechst staining of HSCs identifies a small fraction of bone marrow cells termed side population (SP) cells. SP cells are highly enriched for HSC activity and represent 0.05% of adult nucleated bone marrow cells in mice[46]. SP stem cells have also been identified in hematopoietic compartments of other species such as humans, rhesus monkeys, swine[47-49], and in nonhematopoietic tissues such as skeletal muscle[50, 51], brain[52], and embryonic stem (ES) cells[53]. Biologically, hematopoietic stem cells are defined by their capacity for the long-term engraftment of all blood cell types *in vivo* following bone marrow transplantation and are also functionally defined by assays, such as the SCID-Repopulating assay.

In a most preferred embodiment, the stem cells comprise human CD34+ hematopoietic stem cells.

The stem cells may be cultured in various culture media suitable for mammalian cells. This includes, without limitation, MEM, DMEM, RPMI, IMDM, MEM alpha medium, supplemented with fetal bovine serum and/or human serum and/or horse serum. Serum free media, such as BIT 9500, RTM medium, MyeloCult™ H5100 etc., may also be used, as well as X-vivo and AIMV^{R}. The media are also supplemented with additives such as antibiotics, vitamins, amino acids, etc. The medium may further comprise growth factors and/or cytokines that support, cause or stimulate stem cell expansion or self-regeneration, while essentially maintaining their biological properties. Examples of such factors include SCF, LIF, Flt3 ligand, Tpo, etc.

A human HOXB4 polypeptide designates the wild-type human HOXB4 protein or any biologically active derivative or analog thereof. The sequence of the human HOXB4 protein is disclosed in the literature and available at Genbank, under accession number P17483. The coding nucleic acid sequence has also been disclosed and isolated, and is available at Genbank, under accession number NM024015. A human HOXB4 polypeptide of this invention means any polypeptide comprising all or a portion of the sequence of the wild-type human HOXB4 protein, and retaining the ability (i) to

translocate into cells and (ii) to enhance cell expansion. Typically, the polypeptide is the human protein or any biologically active naturally-occurring variant thereof, such as variants resulting from polymorphism, splicing, post-translational modification, etc. The human HOXB4 polypeptide may comprise a portion of the sequence of the human HOXB4 protein, either alone or fused to a heterologous moiety. Although less preferred, the HOXB4 polypeptide to be used in this invention may also originate from different species.

In a most preferred embodiment, the HOXB4 polypeptide is a human wild-type protein.

As indicated above, a particular feature of this invention resides in the use of a population of donor cells to supply biologically active human HOXB4 polypeptide to stem cells in culture. The donor cells may be of various origin. Most preferably, the donor cell should be an adherent cell, capable of forming monolayers in culture, preferably of mammalian origin. The donor cell should be non-pathogenic and suitable for in vitro or ex vivo culture. The donor cells may be primary cells or an established cell line. It may be autologous with respect to the stem cells, or allogeneic or even xenogeneic. For instance, where the stem cells are of human origin, the donor cells may be of human or other mammalian species, including rodent for instance (e.g., rat, mouse, hamster, etc.). The donor cells may be irradiated prior to their use within the process of this invention, in order to prevent any further undesirable proliferation of these cells.

Examples of suitable donor cells include, without limitation, fibroblasts, stromal cells (isolated from fetal liver, endothelial or bone marrow, etc.) and endothelial cells.

In a most preferred embodiment, the donor cells are capable of sustaining culture of immature human hematopoietic stem cells (i.e., feeder cells). Such cells may, for instance, express growth factors which facilitate stem cell growth or self-regeneration. A most preferred example of such a feeder cell is a stromal cell, particularly a stromal cell line such as MS-5[11, 54-56].

For use in the present invention, the donor cell is genetically engineered to express a human HOXB4 polypeptide. In this regard, the donor cell comprises a nucleic acid encoding a human HOXB4 polypeptide.

The nucleic acid encoding a human HOXB4 polypeptide typically comprises a HOXB4 coding portion and regulatory sequences, causing or facilitating expression of the polypeptide by the donor cells. The regulatory sequences may be selected from a promoter, a transcription terminator, a leader sequence, an enhancer, a translocation enhancer, etc.

Suitable regulatory sequences include promoter sequences functional in the donor cell. Such promoters may be strong or weak, regulated, constitutive or inducible, cell-specific or ubiquitous, etc. They may be of various origins, such as viral promoters, cellular promoters, artificial promoters, etc. Suitable examples include HSV-LTR, CMV, TK, E1A, SV40, PGK, albumin, EF1α, actin, etc.

Other suitable regulatory sequences include sequences causing secretion of the HOXB4 polypeptides by the donor cells. Indeed, because efficient stem cell expansion results from a direct interaction between said cells and the HOXB4 polypeptide, it is best to increase the release of said polypeptide in the culture medium. Various secretion signals may be used in this regard, such as the (murine) Immunoglobulin κ V-J2-C chain leader sequence and the interleukin-7 leader sequence, for instance.

Other suitable regulatory sequences include transcriptional terminators, transcription or translocation enhancers, etc.

In a particular, preferred embodiment, the nucleic acid comprises a HOXB4 coding sequence operably linked to a promoter and a leader sequence.

The nucleic acid may be a DNA or RNA, and may be prepared according to techniques known per se in the art, such as by artificial synthesis, ligation, cleavage, cloning, PCR amplification, etc., or a combination thereof.

The nucleic acid may be integrated into the donor cell genome or autonomously replicating. Various techniques may be used to prepare the recombinant donor cells, i.e., to introduce the recombinant nucleic acid into said cells. Such techniques include, without limitation, virus-mediated infection, plasmid-transfection, electroporation, naked polynucleotide delivery, calcium-phosphate precipitation, liposome-mediated gene transfer, etc.

In a most preferred embodiment, the nucleic acid is introduced into the donor cells by virus-mediated infection. In this respect, different types of viruses may be used, such as retroviruses, lentiviruses, adenoviruses, AAVs, vaccina viruses, herpes viruses, etc. The choice of the virus may be made by the skilled person depending on the nature of the donor cell. In a specific embodiment, the nucleic acid is contained in a retrovirus, more specifically in a lentivirus. As disclosed in the examples, lentiviruses allow very efficient delivery of a nucleic acid into stromal cells and cause efficient and long-term expression thereof.

Viral vectors and recombinant viruses may be produced by techniques known in the art, such as by introducing a recombinant viral vector into a suitable packaging cell, optionally in the presence of a helper plasmid or virus. Viral vectors for use in the present invention are preferably replication-defective, i.e., they are incapable of autonomous replication in a cell. Such vectors (or corresponding recombinant viruses) thus lack at least one functional viral gene encoding a protein involved in viral replication, such as E1 or E4 (adenoviruses), REP or Cap (AAVs), GAG and/or POL (retroviruses), etc.

Methods of producing retroviral vectors (and corresponding recombinant viruses) have been described for instance in WO90/02806, US5,324,645 and WO94/19478. Methods of producing adenoviral vectors (and corresponding recombinant viruses) have been described for instance in WO95/02697 and WO96/22378. Methods particularly

suited for producing lentiviral vectors (and corresponding recombinant viruses) have been described for instance in US5,981,276 and US6,013,516.

The genetically modified donor cells may be cultured in various culture media suitable for cells, including without limitation, MEM, DMEM, RPMI, IMDM, MEM alpha medium, supplemented with fetal bovine serum and/or human serum and/or horse serum. Serum free media, such as BIT 9500, RTM medium, MyeloCult™ H5100, etc., may also be used. The media may also be supplemented with additives such as antibiotics, vitamins, amino acids, etc.

For use in the present invention, the stem cells and donor cells are co-cultured in vitro or ex vivo. The co-culturing may be performed under sterile conditions in any suitable device, such as a plate, flask, bottle, pouch, etc. Preferably, the donor cells are first cultured so as to form a layer of cells. Once the layer is formed, the said layer may be irradiated and the stem cells may be added into the culture, for expansion. It should be noted that the cell populations may be in direct physical contact or not. Direct physical contact means that the cell populations are present in a same culture recipient and may interact physically. This embodiment allows efficient stem cell expansion, and should be followed by a separation step to isolate stem cells from donor cells. The separation step is most preferred for therapeutic uses of the stem cells. It is also possible to perform the co-culture under conditions avoiding direct physical interaction between the cells of the two populations. Indeed, no direct cell-cell contact is needed to obtain the desired enhanced amplification effect, a transfer of growth factors or polypeptides is sufficient. The method can thus be performed by separating the two cell populations with a membrane allowing an exchange of soluble factors, such as soluble polypeptides. A typical example of such a device is a Transwell® Costar 0,4µm culture device, comprising two compartments separated by a membrane having sufficient permeability to allow an exchange of soluble factors.

The culture should be performed for a period of time sufficient to obtain expansion of stem cells. Such cultures may be maintained for instance between 12 hours and 7

weeks, depending on the starting cell populations, typically between 1 and 5 weeks. The presence of the donor cells ensures enhanced amplification. The results disclosed in the present application show that, when cultured on stromal cells genetically engineered to secrete the HOXB4 protein, human hematopoietic stem cells expanded to 5-fold higher levels. Moreover, this expansion was associated with a significantly enhanced stem cell repopulating activity *in vivo* (64% vs. 18%) and the maintenance of the pluripotentiality of the stem cells. The process is particularly advantageous since no genetic modification of the stem cells is needed and, where feeder cells are used, no exogenous cytokine is needed. To our knowledge, this is the first report of a significant amplification of human stem cells without genetic modification thereof or added exogenous cytokines.

Furthermore, in a specific embodiment, instead of donor cells, it should also be possible to use an isolated HOXB4 polypeptide. Indeed, the invention shows, for the first time, that a functional HOXB4 polypeptide can passively translocate into human stem cells and enhance amplification of these cells in culture. Such an isolated polypeptide, typically produced in a recombinant system (e.g., in a baculovirus, mammalian or yeast expression system), could thus be added to the culture medium to enhance amplification.

The expanded cells may be used in many different ways. They may be used for experimental research (e.g., to analyse cell differentiation mechanisms) as well as for industrial research (e.g., screening of compounds causing a modification in their activity or differentiation). They may also be used to produce, in vitro, ex vivo or in vivo, various cell-specific lineages and differentiated, mature cell populations, for research or therapeutic uses. Such differentiated cells may be used in cell therapy, tissue engineering, protein production, etc.

In this regard, a particular object of this invention is a method of producing a pharmaceutical composition comprising stem cells, the method comprising (i) amplifying stem cells as described above, (ii) isolating amplified stem cells and (iii) conditioning said cells in a pharmaceutically acceptable excipient or carrier.

An other object of this invention resides in a method of increasing the stem cell repopulating activity in a human subject, the method comprising (i) collecting a biological sample comprising stem cells from the subject, (ii) co-culturing, ex vivo or in vitro, said stem cells with a population of donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide, under conditions allowing expansion of said stem cells and (iii) administering to the subject said expanded stem cells, said expanded stem cells having increased repopulating activity in said subject. Administration is preferably performed by systemic injection, such as intravenous, intra-arterial, intra-muscular, etc. Administration of stem cells may be performed directly in vicinity of a diseased tissue or organ, such as the liver, pancreas, heart, etc.

Alternatively, the stem cells can be used to produce differentiated cells in vitro or ex vivo. In this respect, in a particular variant, the invention relates to a method of producing differentiated cells in vitro or ex vivo, the method comprising (i) amplifying stem cells as described above, (ii) optionally isolating amplified stem cells, (iii) culturing said amplified stem cells under conditions allowing or favouring differentiation into a pre-determined cell type and (iv) optionally conditioning said differentiated cells in a pharmaceutically acceptable excipient or carrier.

The diluent, carrier or excipient may be any solution, gel, powder, sirup or conditioning suitable for use in the pharmaceutical area, such as isotonic solutions, buffer, stabilizing agents, etc. The cells may be administered at various doses, typically comprised between about 10^5 and 10^8 cells per Kg, more preferably between 10^6 and 10^8 cells per Kg.

The above methods and cell populations may be used to treat various pathological conditions, such as tissue defects or degeneration, cancers, solid tumors, hematopoietic diseases, as well as for the engraftment of a higher number of stem cells corrected by gene therapy, etc.

Further aspects and advantages of this invention will be disclosed in the following experimental section, which should be regarded as illustrative and not limiting the scope of this application.

LEGEND TO THE FIGURES

Fig. 1. Generation and properties of HOXB4 expressing MS-5 cells. ***a***, The various TRIPΔU3EF1α lentiviral vectors contain the central polypurine tract (cPPT), the central triplex sequence (CTS)[39] and the ubiquitous EF1α promoter upstream from the cDNA of interest. ***b***, MS-5 cells were transduced with the indicated construct and stained by immunocytochemistry using a monoclonal antibody against HOXB4 or directly studied with epifluorescence illumination for cells expressing EGFP. ***c***, Nuclear and cytoplasmic extracts from engineered MS-5 cells were analyzed by EMSA using HoxB4 consensus DNA binding sequence. For MS-5/HoxB4 cells, we used nuclear extracts because of the nuclear localization of the homeoprotein (lanes 1-6). For MS-5/SP-HoxB4 cells, we used cytoplasmic extracts because of the cytoplasmic localization of the protein (lanes 7-12). Competition assays were performed using unlabelled oligonucleotide (Oligo) (lanes 3, 6, 9 and 12) and supershift assays, in the presence of monoclonal antibody against HOXB4 (Ab) (lanes 2, 5, 8 and 11). Shifted and supershifted complexes are pointed out by arrows. ***d***, Kinetics of hHOXB4 secretion by irradiated MS-5 engineered cells analyzed by ELISA test.

Fig. 2. HOXB4 protein transfer into human haematopoietic cells. Jurkat cells were co-cultured 48 hours with *a*, MS-5/HoxB4 and *b*, MS-5/SP-HoxB4. Non adherent cells were then labelled by immunocytochemistry using a monoclonal antibody against HOXB4 and revealed by DAB staining.

Fig. 3. Expansion of mature cells and clonogenic progenitors (CFCs) derived from $CD34^+$ cells (n=5). Co-cultures of $CD34^+$ cord blood cells with MS-5/HoxB4 cells (□), MS-5/SP-HoxB4 cells (■) and MS-5/EGFP control cells were performed during 5 weeks. Expansion of *a*, total cells and total $CD34^+$ cells present at 4-5 weeks of co-culture and *b*,

total CFCs determined every week during the time-course of the cultures. Histograms represent fold amplification relative to MS-5/EGFP (amplification in co-cultures with these control cells is 1). Error bars represent standard deviation (±SD).

Fig. 4. Expansion of total cells, CFCs and LTC-ICs derived from $CD34^+CD38^{low}$ cells co-cultured with MS-5/HoxB4 (□), MS-5/SP-HoxB4 (■) or MS-5/EGFP control cells in bulk LTC assays during 5 weeks. Expansion of *a*, total cells and total $CD34^+$ cells at 4-5 weeks of co-culture (n=5) and *b*, total CFCs during the time-course of the cultures (n=5). *c*, Frequency and total number of LTC-ICs present at the end of the 5-week primary co-cultures (n=3): $CD34^+$ cells were sorted then plated for secondary long-term cultures on untransduced MS-5 cells for 5 additional weeks. Frequency of LTC-ICs was determined by limiting dilution analyses (i.e. the reciprocal of the concentration of test cells that gave 37% negative cultures)[15,16]. Total LTC-IC number was obtained by multiplying the frequency of LTC-ICs as determined in secondary cultures by the total number of cells present after the primary long-term co-cultures. Histograms represent fold amplification relative to MS-5/EGFP (amplification in co-cultures with these control cells is 1). Error bars represent ±SD.

Fig. 5. NOD-SCID mice repopulating activity of cells cultured in the presence of hHOXB4. *a*, Percentage of human $CD45^+$ cells in each mouse injected with the total progeny from 5-week co-cultures of 10^4 initial human $CD34^+$ cells with MS-5/EGFP (O) (n=11) or with MS-5/SP-HoxB4 (□) (n=14); $P<0.05$ with Fisher exact test. *b*, Representative FACS profiles of one mouse of each cohort demonstrating multi-lineage human repopulation by engrafted cells. $CD34^+$ cells co-cultured 5 weeks on MS-5/EGFP cells (I-III) or MS-5/SP-HoxB4 (IV-VI) cells showed equivalent lympho-myeloid repopulation in mice 7 weeks post-transplant. Dot plots I and IV define gates for lymphoid and myeloid cells. Dot plots II and V are gated on myeloid cells and dot plots III and VI, on lymphoid cells.

EXAMPLES

METHODS

Primary cells and cell lines. Primary human cord blood CD34-positive cells were obtained with an average enrichment of 85%, as described[38]. Cell sorting was conducted using an EPICS ELITE (Beckman-Coulter) to isolate $CD34^+CD38^{low}$ cells which represent 10-15% of the $CD34^+$ cell population. MS-5 murine stromal cell line was used for co-cultures[11]. Jurkat and MS-5 cell lines were grown in alpha-MEM supplemented with 10% foetal calf serum (FCS).

Long-term culture assays. LTC-IC assays using human haematopoietic cells and 30-Gy pre-irradiated MS-5 cells as feeders were performed in either bulk or limiting dilutions conditions in a long-term culture medium composed of alpha-MEM supplemented with 12.5% FCS, 12.5% horse serum and 10^{-4} M ß-mercaptoethanol. Cells from bulk cultures were counted every week, cloned for progenitor assays and immunophenotyped as described below.

Semi-solid cloning assays. Human clonogenic progenitors were assayed by plating LTC-derived cells in 1 ml standard methylcellulose (Stem Cell Technologies, Vancouver, Canada) supplemented with recombinant human (rhu)-Erythropoietin 2 U/ml, rhu-Interleukin-3 20 ng/ml (Kirin Brewery Co, Tokyo, Japan), rhu-Stem Cell Factor 25 ng/ml, rhu-Granulocyte-Colony Stimulating Factor (CSF) 15 ng/ml (both from AMGEN, Thousand Oaks, California, USA), Granulocyte-Macrophage CSF 5 ng/ml (Schering-Plough, Levallois-Perret, France). Resultant colonies (CFCs) were scored after 15 days incubation.

Cytofluorometry. CD34-fluorescein isothiocyanate (FITC) and CD38-phycoerythrin (PE) antibodies (Immunotech, Villepinte-Roissy-CDG, France) were used for cell sorting. Cells from long-term cultures were immunophenotyped with conjugated monoclonal antibody CD34-allophycocyanin (APC) (Beckton-Dickinson, Le Pont-de-

Claix, France). Presence of human cells in NOD-SCID mice bone marrow was determined using specific human CD45-PE antibody and immunophenotyping was performed with human CD14-PE, CD15-PE, CD19-APC and CD34-FITC specific antibodies (Immunotech).

Construction of HoxB4 vectors. The TRIP vector containing the Enhanced Green Fluorescent Protein (EGFP) cDNA[12] is referred as the EGFP control construct. The HoxB4 and SP-HoxB4 cDNAs were synthesised by PCR amplification. The BamHI-KpnI hHoxB4-containing fragments were then inserted into the TRIP vector plasmid instead of the EGFP cDNA. The HoxB4 construct contained the human HoxB4 cDNA (hHoxB4). The SP-HoxB4 construct contained the murine Ig κ chain leader sequence (Invitrogen, Life Technologies, Cergy-Pontoise, France), a sequence for protein secretion (signal peptide = SP), upstream from the hHoxB4 cDNA.

Lentiviral vector production. Lentiviral vectors were generated by transient calcium phosphate co-transfections of 293T cells by the TRIP vectors and a vesicular stomatis virus envelope expression plasmid, as previously described[39]. Vector particle contents were normalised according to the p24 HIV-1 capsid protein content of the supernatants. Corresponding viral titer determined on MT4 cells was 10^{10}/ml.

MS-5 cell transduction. MS-5 cells, grown to 70% confluence, were transduced with 2.5 μg of recombinant vector p24 per ml of culture for 24 hours. Cells were then washed and cultured for at least 5 serial passages before analyses.

ELISA test. 96-well plates were coated over-night with supernatants from MS-5 engineered cells. After saturation, hHOXB4 protein was detected using the I12 antibody against HOXB4[13,14] (1:100) and a horseradish peroxidase-conjugated secondary rat Ig antibody (Santa-Cruz Biotechnology, Santa-Cruz, California, USA) (1:500). Coloration was performed using o-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich, Saint-Quentin Fallavier, France) and measured at 492 nm on a spectrophotometer.

Immunocytochemistry. The various MS-5 engineered cells or, cytocentrifuged Jurkat cells previously co-cultured 48 hours with MS-5/SP-HoxB4 or MS-5/EGFP cells, were fixed with Zinc-Formal-Fixx (Shandon, Cergy-Pontoise, France) and permeabilised with 0.1% Triton X100. HOXB4 expression was identified using the I12 antibody (1:500), followed by a biotin-conjugated secondary rat Ig antibody and then an Avidin-Biotin horseradish peroxydase complex (Vector, Burlingame, California). Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) chromogen was used to reveal peroxydase activity.

Electrophoretic Mobility Shift Assay. MS-5 cells were lysed as described[40]. Nuclear or cytoplasmic extracts (10 μg) were incubated with the [γ-^{32}P]ATP end-labelled oligonucleotide 5'-CTGCGATGATTGATGACCGC-3' containing the HoxB4 consensus DNA binding sequence[42], then loaded on 6% non-denaturing polyacrylamide gel. For competition assays, the extracts were pre-incubated with an excess (100-fold molar) of unlabelled oligonucleotide. For supershift assays, the extracts were incubated in the presence of antibody against HOXB4.

Mice. Cells derived from 10^4 human CD34$^+$ initial cord blood cells after the 5 week co-culture with either EGFP- or SP-HoxB4-transduced MS-5 cells, were collected and injected into 2.5 Gy-irradiated NOD-LtSz-scid/scid (NOD-SCID) mice. Seven weeks after transplantation, bone marrow nucleated cells from transplanted animals were analysed by flow cytometry for the presence of human CD45$^+$ cells. Mice were considered as positive for human HSCs engraftment when at least 0.1% CD45$^+$ human cells were detected among murine bone marrow cells. Cells from positive mice were further phenotyped using antibodies described in subheading "cytofluorometry".

RESULTS

Efficient hHoxB4 expression in MS-5 cells

The murine stromal cell line MS-5 supports human haematopoiesis in long-term culture[11]. To establish MS-5 cells that constitutively produce hHOXB4 homeoprotein, we

transduced MS-5 cells with hHoxB4 cDNA constructs cloned into the TRIPΔU3EF1α lentiviral vector[12]. These constructs contained either the wild-type hHoxB4 cDNA alone (construct HoxB4) or the hHoxB4 cDNA preceded by a sequence encoding the signal peptide (SP) of the immunoglobulin κ light chain (construct SP-HoxB4) to enhance the secretion of hHOXB4 by MS-5 cells (Fig. 1*a*). As a control, we used a construct containing the EGFP cDNA (construct EGFP). We studied HOXB4 protein expression in the transduced MS-5 cells by immunocytochemical analyses with a rat monoclonal antibody against HOXB4[13,14] and showed that 100% of transduced MS-5 cells stably expressed hHOXB4 (Fig. 1*b*). Cells that had integrated the SP-HoxB4 construct (MS-5/SP-HoxB4 cells) exhibited strong and specific cytoplasmic labelling while cells transduced with the wild-type HOXB4 construct (MS-5/HoxB4 cells) displayed intense nuclear labelling (Fig. 1*b*). Thus, MS-5 engineered cells expressed hHOXB4 protein that was targeted to specific sub-cellular localisations.

To determine whether the hHOXB4 protein produced in the various MS-5 cells retained its capacity to bind its target DNA sequence, we performed Electrophoretic Mobility Shift Assay (EMSA) using an oligonucleotide containing the consensus sequence recognised by HOXB4. We could detect a complex in each of the HoxB4-transduced MS-5 cells used (Fig. 1*c*, lanes 4 and 10), but not in MS-5/EGFP cells (Fig 1*c*, lanes 1-3 and 7-9). This complex was competed by a 100-fold excess unlabelled probe (Fig. 1*c*, lanes 6 and 12) and supershifted by the monoclonal antibody against HOXB4 (Fig. 1*c*, lanes 5 and 11). Thus, hHOXB4 proteins produced by the engineered MS-5 cells retained the ability to bind their target sequence on DNA. Finally, we performed ELISA tests on supernatants of the irradiated MS-5 cells further used for co-culture experiments. We detected hHOXB4 in the supernatant from MS-5/SP-HoxB4, but not in the supernatants from MS-5/HoxB4 or MS-5/EGFP cells (Fig. 1*d*). Altogether, these results indicate that we have established stromal cells able to produce and export functional hHOXB4, thereby supplying a continuous source of this homeoprotein in culture.

hHOXB4 protein secreted by MS-5 cells can enter human haematopoietic cells

To test the ability of the hHOXB4 protein produced by MS-5 cells to translocate into human haematopoietic cells, we co-cultured the various engineered MS-5 cells with Jurkat T-cells. We then performed immunocytochemistry analyses on Jurkat cells and detected mixed nucleo-cytoplasmic HOXB4 labelling in cells that were co-cultured with MS-5/SP-HoxB4 but not in cells that were co-cultured with either MS-5/HoxB4 (Fig. 2) or MS-5/EGFP cells (data not shown). This result demonstrates that hHOXB4 homeoprotein could be efficiently transferred into human haematopoietic cells when co-cultured with MS-5 cells actively secreting this protein.

Amplification of clonogenic progenitors in long-term co-culture of $CD34^+$cells with hHOXB4-producing MS-5 cells

To assess whether the passive transfer of hHOXB4 can lead to an amplification of the human HSC pool, we first performed long-term co-cultures of HSC-enriched cell suspensions ($CD34^+$ cells) with the various MS-5 cells described above. As the MS-5 and MS-5/EGFP cells displayed similar capacities to sustain long-term cultures of human HSCs (data not shown), we used the MS-5/EGFP cells as a control for transduced cells in our experiments. Every week during 5 weeks, we determined (i) the total cell numbers, (ii) the percentage and absolute numbers of $CD34^+$ cells and (iii) the number of clonogenic progenitors (CFCs). After 4-5 weeks, co-cultures with either MS-5/HoxB4 or MS-5/SP-HoxB4 displayed 2.5 to 3 fold higher numbers of total cells as well as $CD34^+$ cells in comparison with cells co-cultured on MS-5/EGFP cells (Fig. 3*a*). Moreover, absolute numbers of CFCs present at the end of the co-culture period with MS-5/HoxB4 or MS-5/SP-HoxB4 cells were 4 times higher than those found in co-cultures with MS-5/EGFP cells (Fig. 3*b*). These data demonstrate that hHOXB4-producing MS-5 cells favour the amplification of human $CD34^+$ and progenitors cells in co-culture.

Amplification of CFCs and LTC-ICs in long-term co-cultures of $CD34^+CD38^{low}$ cells with hHOXB4-producing MS-5 cells

To determine whether primitive cells could also be expanded, we performed long-term co-cultures of $CD34^+CD38^{low}$ cells with the HOXB4-producing MS-5 cells. Following 4-5 weeks of co-cultures with MS-5/SP-HoxB4, amplification rates of total and $CD34^+$ cells were 2.2 ± 0.9 and 3.1 ± 0.9 respectively, as compared to co-cultures with MS-5/EGFP cells (Fig 4*a*). Under the same conditions, CFC amplification was 3 ± 1 and 2.5 ± 0.3 at weeks 4 and 5, respectively (Fig 4*b*). These amplifications could not be obtained when immature $CD34^+CD38^{low}$ cells were co-cultured with MS-5 cells transduced by the HoxB4 construct devoid of a signal peptide (Fig 4*a* and *b*). To study whether the most primitive haematopoietic cells had been efficiently amplified in co-cultures with HOXB4-producing MS-5 cells, we established secondary long-term cultures. $CD34^+$ cells derived from 5-week primary co-cultures were sorted by flow cytometry and plated at limiting dilutions on untransduced MS-5 cells for 5 additional weeks in LTC conditions and the number of LTC-ICs was then determined[15,16]. Co-cultures that had been performed with either MS-5/HoxB4 or MS-5/SP-HoxB4 cells showed an increase in total LTC-IC numbers of 1.7 ± 0.1 and 4.9 ± 0.2 fold, respectively (Fig. 4*c*). The number, size and nature of CFCs (BFU-E vs. CFU-GM) generated from a single LTC-IC that was derived from co-cultures with HOXB4-producing or non-producing MS-5 cells were indistinguishable (data not shown). In conclusion, assuming that the frequency of LTC-ICs in a cord blood-derived $CD34^+CD38^{low}$ cell population is 2 to 5%[11], the overall enrichment in total numbers of LTC-ICs after 5 weeks of primary culture was 18 to 44 fold with MS-5/SP-HoxB4 and 6 to 14 fold with MS-5/HoxB4 cells vs. 3.5 to 8.5 fold with MS-5/EGFP cells. These results represent an *ex vivo* demonstration that hHOXB4 protein delivery into HSCs leads to a high amplification of primitive human haematopoietic cells.

Enhanced engraftment of immune-deficient NOD-SCID mice by hHOXB4-amplified HSCs

Human haematopoietic cells that can engraft NOD-SCID mice bone marrow, the SRCs, are considered to be the most primitive haematopoietic cells experimentally identifiable in humans[17,18]. We co-cultured 10^4 $CD34^+$ cells with MS-5/SP-HoxB4 or MS-5/EGFP cells during 5 weeks and then injected the total cell progeny of the culture

into a sub-lethally irradiated NOD-SCID mouse. Seven weeks post-transplantation, we sacrificed the mice and analysed their bone marrow cell content for the presence of human $CD45^+$ cells. 9/14 (64%) mice injected with the cell progeny of co-cultures with MS-5/SP-HoxB4 vs. 2/11 (18%) mice injected with the cell progeny of co-cultures with MS-5/EGFP, exhibited SRC-derived human cells ($P<0.05$) (Fig. 5*a*). In addition, phenotypic analyses revealed that the expanded SRCs retained full multi-lineage repopulating ability consisting of myeloid $CD14^+/CD15^+$, B-lymphoid $CD19^+$ and $CD34^+$ cells (Fig. 5*b*). Thus, after hHOXB4-induced *ex vivo* expansion, SRCs can engraft NOD-SCID mice without displaying alteration in their differentiation capacity, demonstrating that hHOXB4 induces straightforward amplification of human HSCs.

DISCUSSION

We have established a new protocol for human stem cells *ex vivo* amplification that does not involve genetic alteration of the target cell. We show that the direct delivery of the hHOXB4 protein into haematopoietic cells results in absolute number increase of the most immature haematopoietic cells identifiable in human, LTC-ICs and SRCs, as well as of more mature progenitors. The originality of the approach we describe resides in the epigenetic modification of target cells by providing a source of exogenous protein.

Expansion of human haematopoietic stem cells has concentrated many efforts and two types of strategy towards this aim have arisen: the use of cytokine mixtures and/or additional molecules and the modifications of the HSC genome. As an example of the former strategies[18-20], experiments using a combination of cytokine and the addition of a protein, such as Sonic Hedgehog [21], lead to the expansion of human SRCs. However, it remains unclear if the use of cytokines does not result in the loss of some properties of the amplified stem cells. Modifications of the HSC genome to enhance amplification of said cells has bee reported using different types of proteins, such as AML1-ETO, HOXA-10 and HOXB4[3,4,6,22,23,24,25]. However, such a constitutive expression of proteins in human HSCs, as well as potential risks of insertional mutagenesis[26], might be hazardous for therapeutic applications.

To circumvent these drawbacks, we designed a new approach that avoids both genetic alteration of the target cells and the potential drawbacks of cytokine mixtures. The present invention proposes to use genetically modified donor cells to supply a biologically active hHOXB4 polypeptide to the stem cells during expansion. Certain homeodomain-containing proteins and a number of messenger proteins or peptides such as the synthetic peptide carrier Pep-1[27], transporter peptides derived from *Antennapedia* third helix[28], the HIV transactivation factor TAT[29,30] or Herpes simplex VP22 protein[31] are known to directly translocate into cells and thus be addressed to the cytoplasm or nucleus. The translocation of these proteins follows a non-classical pathway[32-35] as their interaction with cell membrane induces the formation of inverted micelles in which the molecules are trapped. These micelles then open and release the molecules inside the cell[34]. Up to now, most studies on homeoprotein passive translocation have been focused on neuronal and adherent cells[7-10]. The present invention now shows, for the first time, that the HOXB4 protein can efficiently translocate into human stem cells. The present invention also shows, for the first time, that biologically active HOXB4 protein may be produced by donor cells co-cultivated with stem cells, leading to efficient expansion of most primitive cells. The invention further demonstrates, for the first time, that efficient human stem cells may be amplified in the absence of added cytokines using such genetically modified donor cells.

The biological effects of HOXB4 reported here further confirm the amplification-enhancing activity of this polypeptide, and that HOXB4 appears to be a key factor in the determination of definitive haematopoiesis. Moreover, and in contrast to all other reports, we provide evidence that, in stringent conditions *i.e.* without the addition of recombinant cytokines, hHOXB4 protein can be used by itself as an extrinsic growth factor that enhances the number of stem cells while maintaining their biological properties. Finally, HOXB4-amplified HSCs could not be distinguished from non-amplified ones since no difference appeared in the phenotype of cells derived from the various co-culture conditions.

Whereas $CD34^+$ cells exhibited equal responsiveness to amplifying activities of both MS-5/SP-HoxB4 and MS-5/HoxB4 cells, $CD34^+CD38^{low}$ cells appeared much more

insensitive to MS-5/HoxB4 cells. This difference in the behaviour of the two cell populations could be related to dose-dependent variations in their HOXB4 requirement for amplification: among $CD34^+$ cells, $CD34^+CD38^{low}$ cells seemed less sensitive than $CD34^+CD38^{high}$ cells to the action of MS-5/HoxB4 cells that produced undetectable amounts of hHOXB4 protein in culture supernatants.

The HOXB4-dependent cell expansion might be related to the hHOXB4 transfer into haematopoietic cells (direct effect) or to putative changes of HOXB4-expressing MS-5 cells (indirect effect). However, several observations argue against the latter mechanism: (i) similar effects on $CD34^+$ cells are obtained with MS-5 cells expressing HOXB4 or SP-HOXB4 while HOXB4 sub-cellular localisation in the MS-5 cells is completely different, (ii) phenotypic characteristics (morphology, growth rate) of MS-5 cells are not modified by any of the transduction performed, and (iii) $CD34^+CD38^{low}$ cells are much less sensitive to the action of non actively secreted hHOXB4, thus providing an internal control against an indirect effect of HOXB4 through MS-5-cell changes. These observations thus strongly argue in favour of a direct involvement of HOXB4 in the HSC amplification we obtained.

In conclusion, the present report shows that the direct delivery of HOXB4 into human HSCs leads to *ex vivo* expansion of these cells. Both recently published data that demonstrate haematopoietic potential of human embryonic stem cells[37] and our results provide bases for the development of new therapeutic strategies that include HOXB4-mediated amplification of human haematopoietic stem cells and of other stem cells.

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CLAIMS

1. A method of amplifying human stem cells ex vivo or in vitro, the method comprising co-culturing human stem cells with a population of donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide.

2. A method of enhancing amplification of human stem cells, the method comprising co-culturing, ex vivo or in vitro, human stem cells with a population of donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide, said human HOXB4 polypeptide causing enhanced amplification of human stem cells.

3. The method of claim 1 or 2, wherein the stem cells are hematopoietic stem cells.

4. The method of any of claims 1 to 3, wherein the donor cells are stromal cells.

5. The method of any of claims 1 to 4, wherein the human HOXB4 polypeptide is the wild-type human HOXB4 protein.

6. The method of any of claims 1 to 5, wherein the recombinant nucleic acid comprises a leader sequence causing secretion of the HOXB4 polypeptide.

7. The method of any of claims 1 to 6, wherein the stem cells and donor cells are physically separated in said co-culture by a membrane allowing exchange of soluble factors.

8. A composition of matter comprising human stem cells and donor cells, said donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide.

9. A composition of claim 8, comprising human hematopoietic stem cells and stromal cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide.

10. A recombinant lentivirus, wherein said lentivirus encodes a human HOXB4 polypeptide.

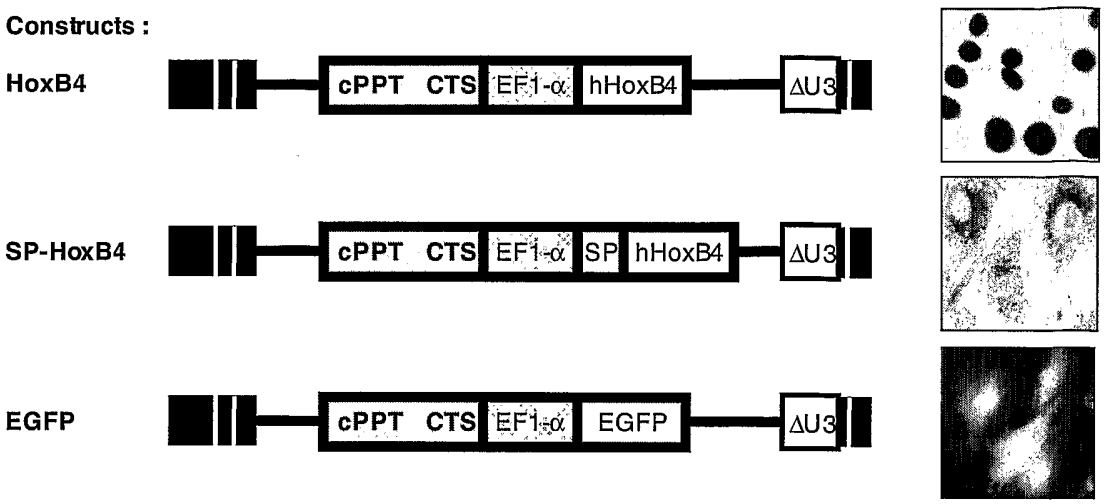
11. A recombinant donor cell, wherein said donor cell is an irradiated adherent cell capable of forming monolayers in culture, and wherein said donor cell comprises a recombinant nucleic acid encoding a human HOXB4 polypeptide.

12. A method of increasing the stem cell repopulating activity in a human subject, the method comprising (i) collecting a biological sample comprising stem cells from the subject, (ii) co-culturing, ex vivo or in vitro, said stem cells with a population of donor cells comprising a recombinant nucleic acid encoding a human HOXB4 polypeptide and expressing said human HOXB4 polypeptide, under conditions allowing expansion of said stem cells and (iii) administering to the subject said expanded stem cells, said expanded stem cells having increased repopulating activity in said subject.

13. A method of producing a pharmaceutical composition comprising human stem cells, the method comprising (i) amplifying human stem cells according to the method of any one of claims 1 to 7, (ii) isolating amplified stem cells and (iii) conditioning said cells in any pharmaceutically acceptable excipient or carrier.

14. The use of a recombinant cell of claim 11 for the amplification in vitro or ex vivo of human stem cells.

15. A method of producing differentiated cells in vitro or ex vivo, the method comprising (i) amplifying stem cells as described above, (ii) optionally isolating amplified stem cells, (iii) culturing said amplified stem cells under conditions allowing or favouring differentiation into a pre-determined cell type and (iv) optionally conditioning said differentiated cells in a pharmaceutically acceptable excipient or carrier.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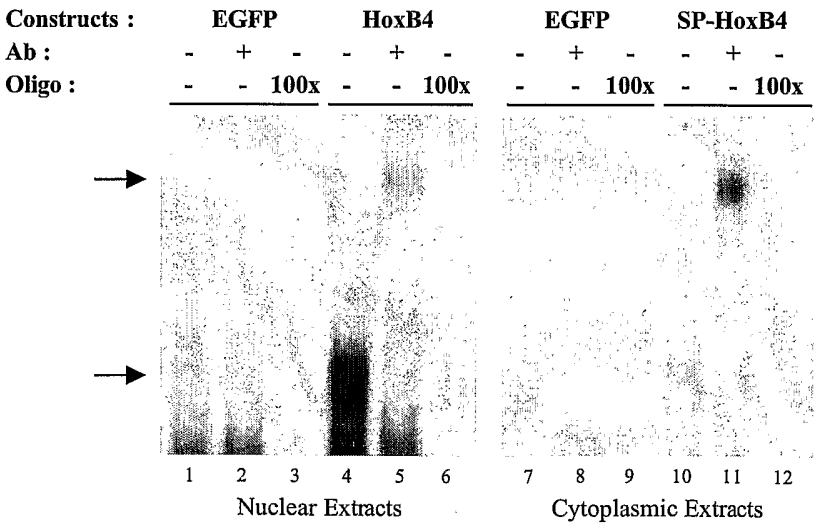
SP-HoxB4
cPPT CTS EF1-α SP hHoxB4 ΔU3
EGFP
cPPT CTS EF1-α EGFP ΔU3

a *b*


Constructs : EGFP HoxB4 EGFP SP-HoxB4
Ab : - + - - + - - + - - + -
Oligo : - - 100x - - 100x - - 100x - - 100x
1 2 3 4 5 6 7 8 9 10 11 12
Nuclear Extracts
Cytoplasmic Extracts

c

Fig. 1

d

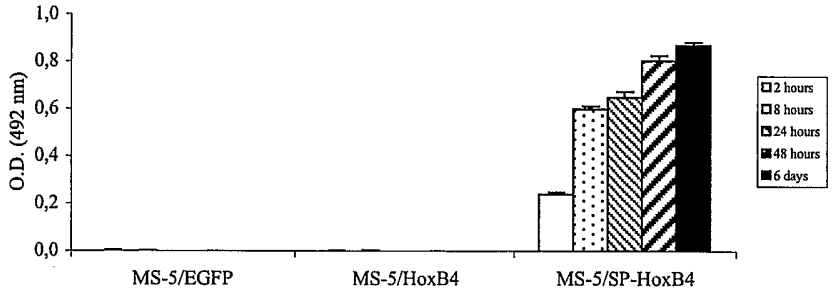
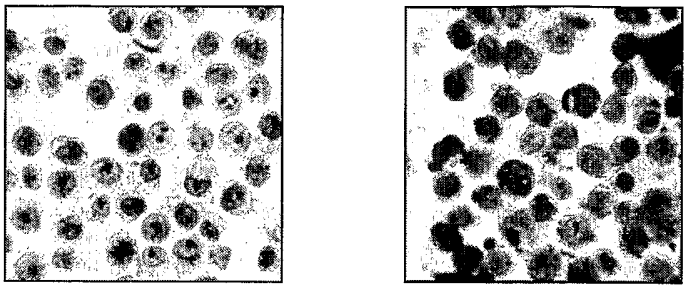

1,0
0,8
0,6
0,4
0,2
0,0
O.D. (492 nm)
MS-5/EGFP
MS-5/HoxB4
MS-5/SP-HoxB4
2 hours
8 hours
24 hours
48 hours
6 days

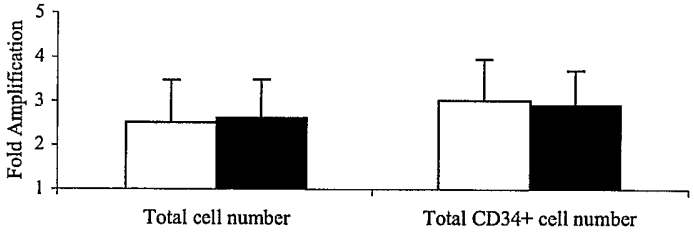
Fig. 1



a *b*

Fig. 2

a


Fold Amplification
5
4
3
2
1
Total cell number
Total CD34+ cell number

b

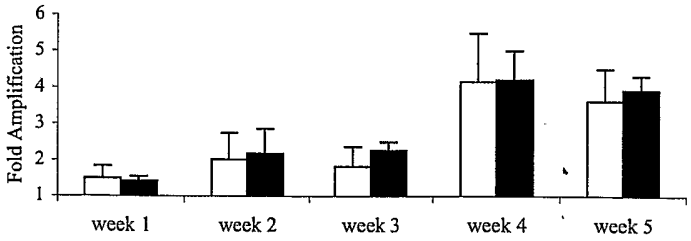

Fold Amplification
6
5
4
3
2
1
week 1
week 2
week 3
week 4
week 5

Fig. 3

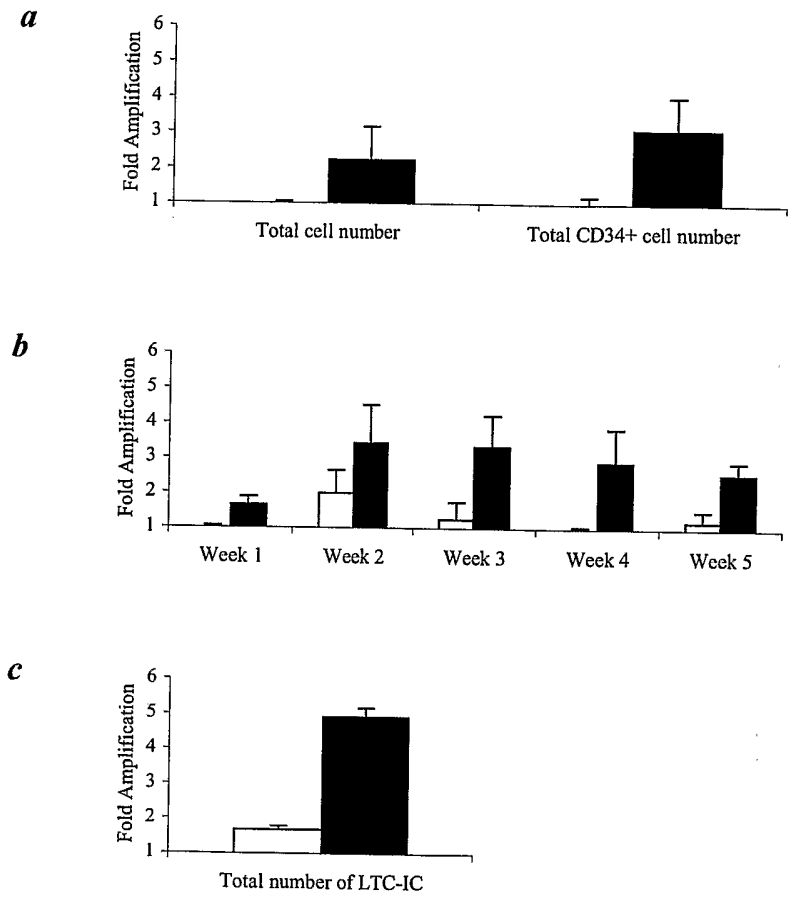
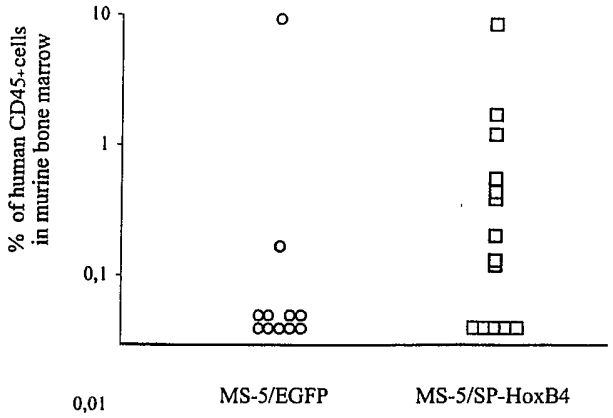

a
Fold Amplification
6
5
4
3
2
1
Total cell number
Total CD34+ cell number
b
Fold Amplification
6
5
4
3
2
1
Week 1
Week 2
Week 3
Week 4
Week 5
c
Fold Amplification
6
5
4
3
2
1
Total number of LTC-IC

Fig. 4

a


% of human CD45+cells in murine bone marrow
10
1
0,1
0,01
MS-5/EGFP
MS-5/SP-HoxB4

b

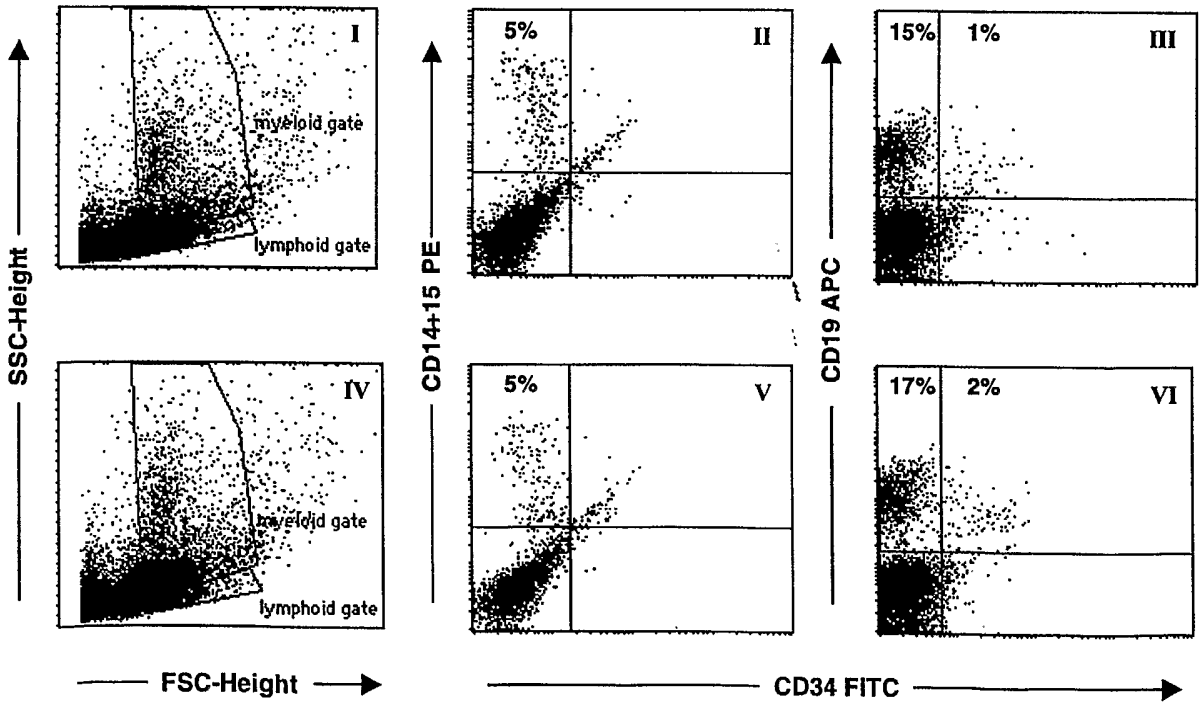

SSC-Height
I
myeloid gate
lymphoid gate
IV
myeloid gate
lymphoid gate
FSC-Height
CD14+15 PE
5%
II
5%
V
CD19 APC
15%
1%
III
17%
2%
VI
CD34 FITC

Fig. 5

INTERNATIONAL SEARCH REPORT

PCT/IB 02/04319

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C07K14/475

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMSELLEM S ET AL: "Ex vivo amplification of human hematopoietic stem cells by using passive transduction of HOXB4 homeoprotein." EXPERIMENTAL HEMATOLOGY (CHARLOTTESVILLE), vol. 30, no. 6 Supplement 1, June 2002 (2002-06), page 124 XP002229463 31st Annual Meeting of the International Society for Experimental Hematology;Montreal, Quebec, Canada; July 05-09, 2002, June, 2002 ISSN: 0301-472X abstract --- -/--	1-9, 12-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2003	13.06.03

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Nichogiannopoulou, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BUSKE C ET AL: "Constitutive expression of the homeobox transcription factor HOXB4 amplifies human primitive hematopoietic progenitor cells in vitro and in vivo" EUROPEAN JOURNAL OF CANCER, PERGAMON PRESS, OXFORD, GB, vol. 37, September 2001 (2001-09), page S87 XP004381597 ISSN: 0959-8049 abstract ---	1
A	US 5 837 507 A (HUMPHRIES R KEITH ET AL) 17 November 1998 (1998-11-17) column 8, line 5 - line 18; claim 7 ---	1
A	SAUVAGEAU GUY ET AL: "Overexpression of HOBXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo." GENES & DEVELOPMENT, vol. 9, no. 14, 1995, pages 1753-1765, XP009005048 ISSN: 0890-9369 cited in the application the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

PCT/IB 02/04319

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 12-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9, 12-15

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9, 12-15

   Methods and compositions of matter relating to the coculture of donor cells expressing recombinant human HOXB4 and human stem cells.

2. Claim : 10

   A recombinant lentivirus encoding human HOXB4.

3. Claim : 11

   Irradiated adherent cells expressing recombinant human OXB4.

INTERNATIONAL SEARCH REPORT

PCT/IB 02/04319

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5837507 A	17-11-1998	NONE	